Differentially expressed circRNA and mRNA profiles of neural stem cells with radiation treatment

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Research Article

Keywords: neural stem cell, circular RNA, radiation treatment

Posted Date: July 22nd, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1831154/v1

Abstract

Background: Spine metastasis is common but highly problematic in clinical oncology practice. Radiotherapy plays an important role in treatment of spine metastasis but damages nervous tissue, especially neural stem cells (NSCs), and leads to radiation-induced myelopathy. Circular RNA (circRNA) is a type of noncoding RNA that responds to external stimuli and regulates cellular functions. However, the mechanism by which radiotherapy affects NSCs and the role of circRNAs in this process are still unclear.

Methods: circRNAs and mRNAs in NSCs treated with or without radiation were detected using next-generation sequencing. RT-PCR assays were performed to confirm the sequencing results and differentially expressed circRNAs. Bioinformatic analyses were conducted to identify the critical circRNAs and mRNAs, as well as the enriched functions and pathways. Moreover, a circRNA-miRNA-mRNA network was constructed to investigate the possible regulatory mechanism.

Results: A total of 421 differentially expressed circRNAs and 1602 differentially expressed mRNAs were identified in NSCs after radiotherapy. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses of the differentially expressed mRNAs and of the host genes of the differentially expressed circRNAs were performed, and several key signaling pathways, such as the MAPK signaling pathway, were identified. Moreover, a circRNA-miRNA-mRNA network focusing on the MAPK signaling pathway was constructed and predicted that chr5:127160496|127165240 could be a critical circRNA in the mechanism underlying the response of NSCs to radiation treatment.

Conclusion: Our findings reveal the differentially expressed circRNA and mRNA profiles of NSCs after radiotherapy and suggest that circRNAs may contribute to the pathogenesis of radiation-induced myelopathy.

Trial registration: N/A

Background

Bone is one of the most common organs affected by cancer metastasis [1]. Based on postmortem studies, bone metastasis is found in approximately 70% of patients with breast or prostate cancer. Bone metastasis reduces the quality of life of patients, leading to pain, limited mobility or even malignant spinal compression [2]. To address this highly problematic issue in clinical oncology practice, many therapeutic schedules, including radiotherapy, have been proposed in recent years [3].

Radiotherapy is widely used for clinical treatment of cancer as well as bone metastasis. It helps to inhibit the growth of tumors and limits bone metastatic lesions, which in turn reduces pain symptoms and improves quality of life [4]. However, radiotherapy may cause radiation-induced myelopathy, especially spine bone metastasis [5]. Recent studies have demonstrated that radiation treatment damages the functions of neural stem cells (NSCs), which is one of the key pathogeneses of radiation-induced myelopathy [6]. However, the detailed mechanism is largely unclear.

NSCs are self-renewing and multipotent cells in nervous tissue. NSCs remain quiescent most of the time but can differentiate into multiple cell lineages, including neurons, astrocytes and oligodendrocytes, after external stimulation [7]. These differentiation abilities are regulated by various mechanisms, and regulatory dysfunction leads to abnormal NSC differentiation and subsequent nervous system disorders [8].

Circular RNA (circRNA) is a member of the noncoding RNA family. circRNA lacks both the 3’ and 5’ ends and the poly A tail, which allows it to form a special covalently closed continuous loop [9]. circRNAs show great potential in regulating molecular expression through several mechanisms, including alternative splicing, miRNA sponging and RNA binding protein interactions [10]. For example, Reut et al. reported that circMbl regulates MLB expression through pre-mRNA splicing [11]. Moreover, Long Yu demonstrated that circRNA_0016624 enhances BMP2 expression by sponging microRNA-98 in postmenopausal osteoporosis [12]. Specifically, Yang et al. demonstrated that circRNAs play important roles in regulation of cell functions, including NSCs [13]. However, whether circRNAs contribute to radiation-induced myelopathy is still unknown.

In this study, we detected differentially expressed circRNA and mRNA profiles in NSCs after radiation treatment. In addition, Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were performed based on the differentially expressed mRNAs and the host genes of circRNAs. Focusing on the MAPK signaling pathway, a circRNA-miRNA-mRNA network was constructed to investigate the possible mechanism underlying the response of NSCs to radiation treatment.

Methods

Cell isolation and culture

Briefly, the spinal cord tissues of neonatal male Sprague-Dawley rats, which were provided by the Laboratory Animals of Sun Yat-Sen University, were digested into single cells by repeated trituration with fire-polished Pasteur pipettes, according to a previously described method for spinal cord stem cells [30]. The cells were centrifuged and then suspended in Dulbecco’s modified Eagle’s medium (DMEM)/F12 supplemented with 2% B27 supplement, 5 μg/ml heparin, 20 ng/ml bFGF and 20 ng/ml EGF. All cells were cultured at 37°C in a humidified atmosphere with 5% CO_2, and the medium was changed every 3 days. The cells were used for experiments without passage. This study was approved by the Committee for the Care and Use of Laboratory Animals of Sun Yat-Sen University, Guangzhou, China.

Radiation treatment

Cultured NSCs were centrifuged and seeded into 4% poly-L-polylysine-coated 6-well plates. The cells were immediately treated with radiation at a dose of 16 Gy. Cultured NSCs that received no radiation were used as a control. All NSCs were used for experiments immediately after treatments.

circRNA and mRNA sequencing expression profiling

RNA samples were immediately extracted from NSCs after radiation treatment. Total RNA was extracted from three radiation-treated NSCs and three corresponding control NSCs using TRIzol reagent and purified using NucleoSpin RNA cleanup kits according to the manufacturer's instructions. RNA integrity was determined by formaldehyde denaturing gel electrophoresis. Ribosomal RNA in total RNA was depleted and then digested by RNase R to remove the linear RNA. The residual RNA was reverse-transcribed into cDNA using a PrimeScript™ RT reagent Kit. End repair and adaptor ligation were performed using Illumina's TrueSeq Total RNA Library Prep Kit. cDNA fragments 250–300 bp in size were separated and then PCR-amplified for approximately 20 cycles to build the library. After further purification and detection, single-end RNA-Seq was performed using an Illumina HiSeq 2000 sequencer. RNA-Seqshort reads were aligned to the rat reference genome (mRatBN7.2) using TopHat (version 2.1.1). The RNA-Seq data were initially filtered through TopHat by mapping to the reference genome. The canonical splicing sites were detected, and the reads were mapped. Then, the unmapped reads were processed using BWA methods. circRNAs demonstrating a $\log_2$ ratio (radiation-treated NSCs/NSCs) of $\geq|1|$ and P-values of ≤ 0.05 were regarded as significantly differentially expressed. The false discovery rate (FDR) was used to correct for false positives.

circRNA identification

Total RNA was extracted and reverse-transcribed into cDNA as described above. Genomic DNA was extracted using a PureLink™ Genomic DNA Mini Kit. Two sets of primers (an outward-facing set and an opposite-directed set) for circRNA and GAPDH were designed using Primer Express software version 5.0. RT-PCR was performed with a Power SYBR® Green RNA-to-CTTM One-Step Kit using a Stratagene Mx3005P Real-Time PCR detection system. After gel purification using a QIAquick Gel Extraction Kit, the RT-PCR product was sequenced using the Sanger method to confirm head-to-tail splicing.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted and reverse-transcribed into cDNA as described above. qRT-PCR assays were performed using SYBR Premix Ex TaqTM in a LightCycler 480 PCR System. All the data were normalized to GAPDH expression. The relative circRNA and mRNA expression levels were analyzed using the $2^{-\triangle\triangle Ct}$ formula. The forward and reverse primers for each gene are presented in Supplemental Table 6.

Bioinformatics analysis

GO and KEGG analyses were performed using DAVID (version 6.8) [31, 32]. Differentially expressed mRNAs and host genes of differentially expressed circRNAs were divided into three subgroups by GO analysis, namely, BP, CC and MF. The related signaling pathways of these genes were determined via KEGG enrichment analysis.

To determine the primary functions of the differentially expressed genes, pathway analysis of the differentially expressed genes was carried out using the KEGG database. Furthermore, coding-noncoding gene coexpression (CNC) networks were constructed for the HDMSC or ASMSC data based on the results

of correlation analyses between differentially expressed mRNAs and lncRNAs (Pearson correlation coefficients > 0.99 or ≤ 0.99). P values < 0.05 were considered statistically significant.

We utilized miRanda and RNAhybrid as tools to predict the related target miRNAs and mRNAs. Ultimately, graphs of circRNA-miRNA-mRNA sharing meaningful correlation interaction networks were drawn using Cytoscape (version 3.3).

Statistical analysis

Statistical analysis was performed with SPSS software. All data are expressed as the means ± SD. P values < 0.05 were considered to indicate a significant difference.

Results

Differential expression profiling of circRNA and mRNA in NSCs treated with radiation

The circRNA and mRNA expression profiles of NSCs before and after radiation treatment were detected by sequencing. Heatmaps of differentially expressed circRNAs and mRNAs are shown in Fig. 1. A total of 421 differentially expressed circRNAs were identified in NSCs treated with radiation compared to NSCs without radiation treatment. Among these differentially expressed circRNAs, 21 circRNAs were upregulated, and 400 circRNAs were downregulated. The top 10 differentially expressed circRNAs are shown in Supplemental Tables 1 and 2. In addition, a total of 1602 differentially expressed mRNAs were identified, with 641 upregulated mRNAs and 961 downregulated mRNAs. The top 10 differentially expressed mRNAs are shown in Supplemental Tables 3 and 4.

GO and KEGG analyses of the host genes of differentially expressed circRNAs

Under the assumption that circRNA function would be related to the known function of the host linear transcripts, we analyzed the host genes of differentially expressed circRNAs using DAVID (Database for Annotation, Visualization and Integrated Discovery) bioinformatics resources to investigate the possible mechanism by which NSCs are affected by radiation treatment. GO analysis was performed to classify these host genes of differentially expressed circRNAs into 3 domains: biological process (BP), molecular function (MF), and cellular component (CC). The GO terms of BP included signaling, response to stimulus and cellular process (Fig. 2a). The CC terms were enriched in cell junctions, membranes and nucleoids (Fig. 2b). Terms relating to signal transducer activity and molecular transducer activity were enriched in the MF GO analysis (Fig. 2c). Moreover, the functional pathways of these host genes were determined by KEGG enrichment analysis, which indicated that several key pathways, such as the MAPK signaling pathway and miRNA in cancer signaling, were involved in the reaction of NSCs to radiation treatment (Fig. 2d).

GO and KEGG analysis of differentially expressed mRNAs

A cell's mRNA expression profile reflects its status and function. We studied the change in NSCs treated with radiation by analyzing the GO terms of differentially expressed mRNAs. GO analysis of the differentially expressed mRNAs in radiation-treated NSCs revealed that BP terms were enriched in signaling activity and stimulus response (Fig. 3a). Similar to the circRNA results, the CC terms were again enriched in cell junctions, membranes and nucleoids (Fig. 3b). In MF, molecular activity and transducer-related terms were enriched in the GO analysis (Fig. 3c). In addition, KEGG analysis was performed to study the involved signaling pathways. Several related pathways, including the MAPK signaling pathway and cancer pathway, were enriched among the top 20 signaling pathways (Fig. 3d).

circRNA-miRNA-mRNA analysis of differentially expressed circRNAs and mRNAs in the MAPK signaling pathway

Many studies have demonstrated that the MAPK signaling pathway is the critical pathway in regulating NSC function and differentiation. As shown by KEGG analysis of both the differentially expressed mRNAs and the host genes of the differentially expressed circRNAs, the MAPK signaling pathway was enriched as one of the most significant functional pathways, indicating the importance of the MAPK signaling pathway in radiation-treated NSCs (Figs. 2 and 3). Therefore, a circRNA-miRNA-mRNA network was constructed based on the differentially expressed mRNAs in the MAPK signaling pathway (Fig. 4). This network included six circRNAs, 46 miRNAs and 13 mRNAs, and the top 5 circRNA-miRNA-mRNA connections in the MAPK signaling pathway are shown in Supplemental Table 5. In this network, chr5:127160496|127165240 possessed more binding sites and target miRNAs, which had a relationship with all the mRNAs in the MAPK signaling pathway, suggesting its role in NSCs with radiation treatment.

Validation of differentially expressed circRNAs and mRNAs in the MAPK signaling pathway

Another nine NSCs were isolated for validation assays. To confirm the differential expression of circRNAs and mRNAs in the circRNA-miRNA-mRNA network of the MAPK signaling pathway, PCR assays with Sanger sequencing were performed. As shown in Fig. 5a, all 6 differentially expressed circRNAs in the network were amplified using outward-facing primers and cDNA as templates and could not be amplified using genomic DNA. These results confirm the circular form of the 6 circRNAs. In addition, the conjunctive site was identified by Sanger sequencing. Finally, the differential expression levels of these circRNAs and mRNAs were confirmed using qRT-PCR assays, which were consistent with the sequencing results (Fig. 5b and c).

Discussion

In this study, we investigated the circRNA and mRNA expression profiles of NSCs treated with radiation. Hundreds of differentially expressed circRNAs and mRNAs related to radiation treatment were identified in NSCs. In addition, using GO and KEGG enrichment analyses, the functions and signaling pathways of these differentially expressed circRNAs and mRNAs were determined, and the MAPK signaling pathway was identified as the key pathway in NSCs treated with radiation. Moreover, a circRNA-miRNA-mRNA

network based on the MAPK signaling pathway was constructed to investigate the possible regulatory mechanism by which NSCs are affected by radiation treatment.

Bone metastasis, especially spine metastasis, is one of the most common and thorough problems in orthopedic clinics. The therapeutic schedule for spine metastasis is the focus of clinical research, but several controversies still need to be addressed [14]. Previously, we demonstrated that intraoperative radiotherapy can effectively relieve pain, achieve good local control of spine metastasis and improve quality of life [4]. However, radiation treatment may cause radiation-induced myelopathy by damaging NSCs [6]. NSCs are undifferentiated stem cells that are defined by their replicative potential, long-term self-renewal, and ability to differentiate into multiple neuronal and glial cell types [15]. Several studies have demonstrated that radiation treatment increases apoptosis of NSCs and inhibits the self-renewal and differentiation potential of NSCs [16, 17]. However, the mechanism by which radiation treatment affects NSCs is largely unknown.

circRNA, a type of noncoding RNA, has recently been recognized as a new class of functional molecule. Due to its circular configuration through a typical 5' to 3'-phosphodiester bond, circRNAs remain stable in cells and take an active part in regulation of cell functions via transcriptional and posttranscriptional activities [9]. In particular, circRNAs contribute greatly to the self-renewal and differentiation capacities of stem cells [18]. The circRNA profile of NSCs during differentiation has been detected previously [13]. In addition, the circRNA HIPK2 has been shown to regulate NSC differentiation into neurons [19]. A previous study revealed that radiation altered the circRNA profile [20], and thus, we speculate that radiation treatment may damage NSC function through intracellular circRNAs. In this study, for the first time to the best of our knowledge, we determined that the circRNA profile of NSCs was significantly changed by radiation treatment. A total of 421 circRNAs were differentially expressed in NSCs after radiation treatment compared to NSCs without radiation treatment. These results indicate that radiation treatment may affect NSCs by altering their circRNA profile, and these 421 differentially expressed circRNAs may play important roles in regulating NSC function during radiation treatment.

NSC status and function are represented by their gene expression profile [21]. To investigate the altered function of NSCs after radiation treatment and the possible mechanism, we analyzed the differentially expressed mRNAs and host genes of differentially expressed circRNAs using GO and KEGG databases. In GO analysis, terms including signaling and response to stimulus were enriched, indicating the effect of radiation treatment on NSCs. Specifically, among both the differentially expressed mRNA and the host genes of differentially expressed circRNA, we found that the MAPK signaling pathway was enriched as the key pathway, indicating that the MAPK signaling pathway plays an important role in NSCs after radiation treatment. MAPK signaling pathways are widely involved in regulation of cell function, including in NSCs [22, 23]. Previous studies have demonstrated that radiation therapy upregulates the expression of genes in the MAPK signaling pathway [24]. Moreover, the activation level of the MAPK signaling pathway has been reported to be under the control of a large number of circRNAs [25–27]. From these results, we suggest that radiation therapy may alter the circRNA expression profile, which in turn regulates the activation level of the MAPK signaling pathway and affects the function of NSCs.

Accumulating evidence has indicated that circRNAs might regulate the function of miRNAs acting as competing endogenous RNAs (ceRNAs) [28]. To study the detailed mechanism by which circRNAs regulate the MAPK signaling pathway in radiation-treated NSCs, a circRNA-miRNA-mRNA network was constructed using differentially expressed circRNAs and differentially expressed mRNAs in the MAPK signaling pathway. Several miRNAs in this network, such as miRNA204, have been demonstrated to be key regulators of NSC function [29]. In addition, we found in this network that chr5:127160496|127165240 has the highest number of targets per 100b. In addition, it may bind to a total of 34 predicted miRNAs and regulate all 13 differentially expressed mRNAs in the MAPK signaling pathway in radiation-treated NSCs. Therefore, we suggest that chr5:127160496|127165240 may be a critical circRNA in NSCs after radiation therapy. Whether and how circRNAs regulate the expression of these differentially expressed mRNAs in the MAPK pathway through miRNAs still need to be addressed in future studies.

Conclusions

In this study, we investigated the differentially expressed circRNA and mRNA profiles of NSCs treated with radiation. The possible affected functions and mechanisms were analyzed using bioinformatic methods. However, some limitations still exist in this study. For example, which circRNA plays a key role during radiation therapy still needs to be confirmed. In addition, how key circRNAs function and regulate NSC function is unknown. Further studies should be conducted to answer these questions.

Abbreviations

neural stem cell, NSC

circular RNA, circRNA

biological process, BP

molecular function, MF

cellular component, CC

competing endogenous RNAs, ceRNAs

Declarations

Ethics approval and consent to participate

All experimental protocols were approved by the Committee for the Care and Use of Laboratory Animals of Sun Yat-Sen University, Guangzhou, China. All methods were carried out in accordance with relevant guidelines and regulations. All methods are reported in accordance with ARRIVE guidelines (https://arriveguidelines.org) for the reporting of animal experiments.

Consent for publication

Not applicable.

Availability of data and materials

All the data are available from the authors. The datasets generated and/or analyzed during the current study are available in the Sequence Read Archive (SRA) repository, ID 736187 - BioProject - NCBI (nih.gov).

Competing interests

The authors declare that they have no competing interests.

Funding

This study was financially supported by the National Natural Science Foundation of China (81703163 to Keng Chen and 31700884 to Shan Wang), Guangdong Province Science and Technology Plan (2017A020215070 to Shan Wang) and Sun Yat-sen University Young Teachers Cultivation Project (17ykpy42 to Shan Wang).

Authors' contributions

HJC, WS, and CK designed the research studies, conducted the experiments, analyzed the data, and wrote the manuscript.

XZY and WP conducted the experiments, analyzed the data, and wrote the manuscript.

WP HJC and CZP conducted experiments.

All authors have read and approved the manuscript and ensure that this is the case.

Acknowledgments

Not applicable.

Conflict of interest statement

Each author certifies that he or she has no commercial associations that might pose a conflict of interest in connection with the submitted article.

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Figures

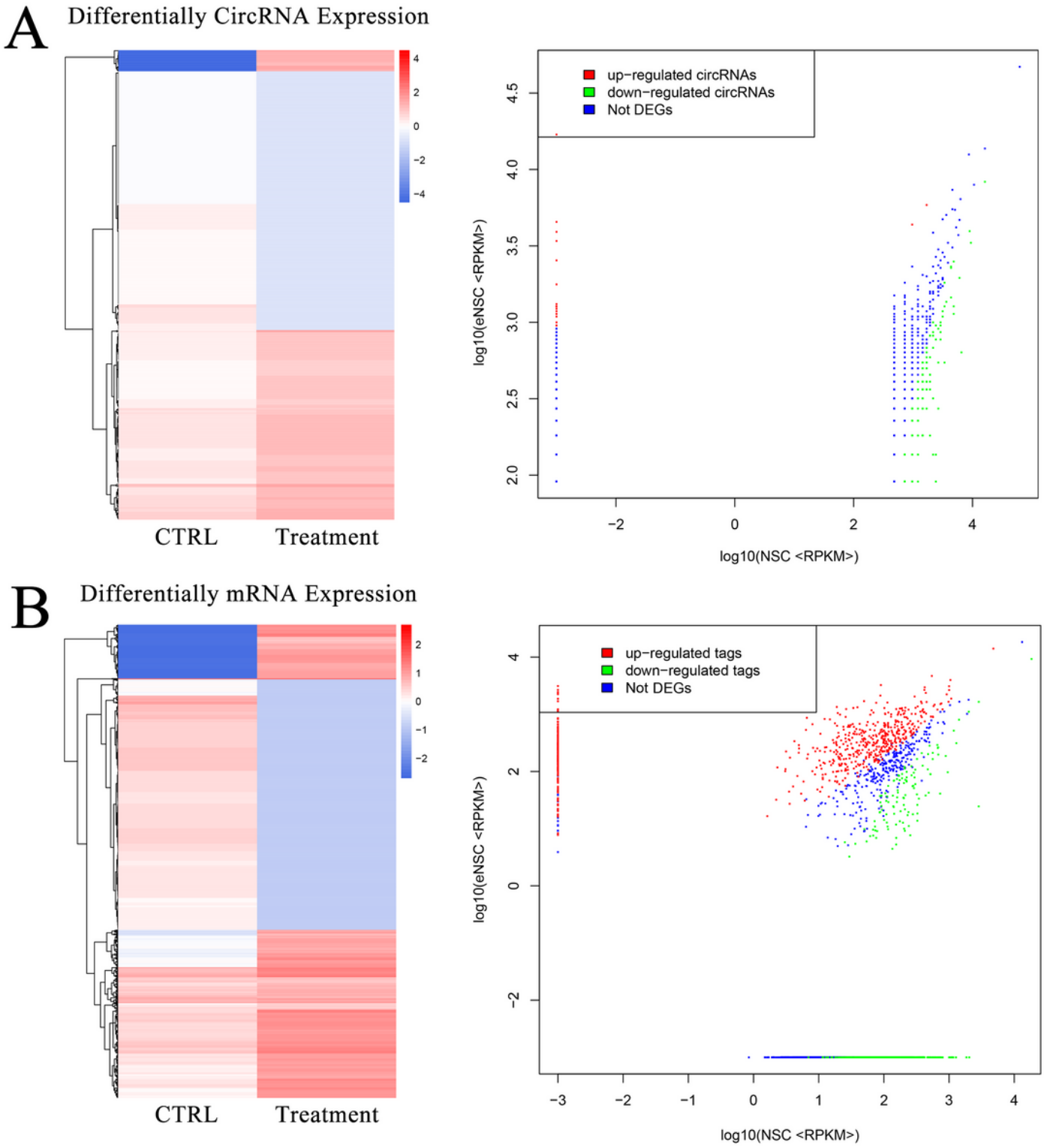

Figure 1

Differentially expressed circRNA and mRNA profiles. (a) A total of 421 circRNAs were differentially expressed in NSCs treated with radiation (treatment group) compared to NSCs without treatment (CTRL group). Hierarchical clustering and scatter plot analysis showed a distinguishable circRNA expression profile. **(b)** A total of 1602 mRNAs were differentially expressed in NSCs treated with radiation (treatment

group) compared to NSCs without treatment (CTRL group). Hierarchical clustering and scatter plot analysis showed a distinguishable mRNA expression profile.

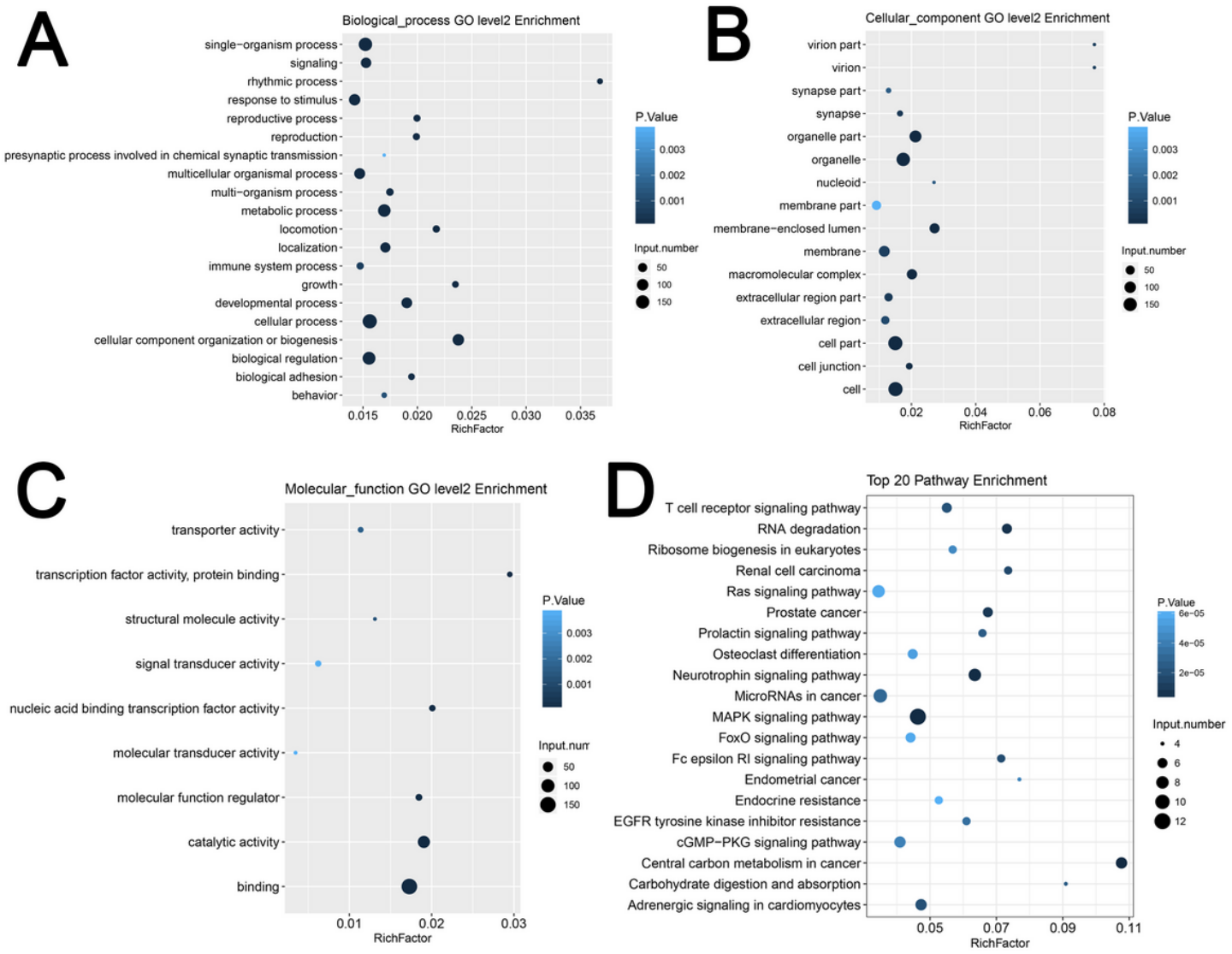

Figure 2

GO and KEGG analyses of the host genes of differentially expressed circRNAs. (a) The top 20 biological process terms revealed by GO analysis are shown. **(b)** The molecular function terms with significant differences in the GO analysis are shown. **(c)** The cellular component terms with significant differences in the GO analysis are shown. **(d)** The top 20 functional pathways of circRNA host genes were determined by KEGG enrichment analysis.

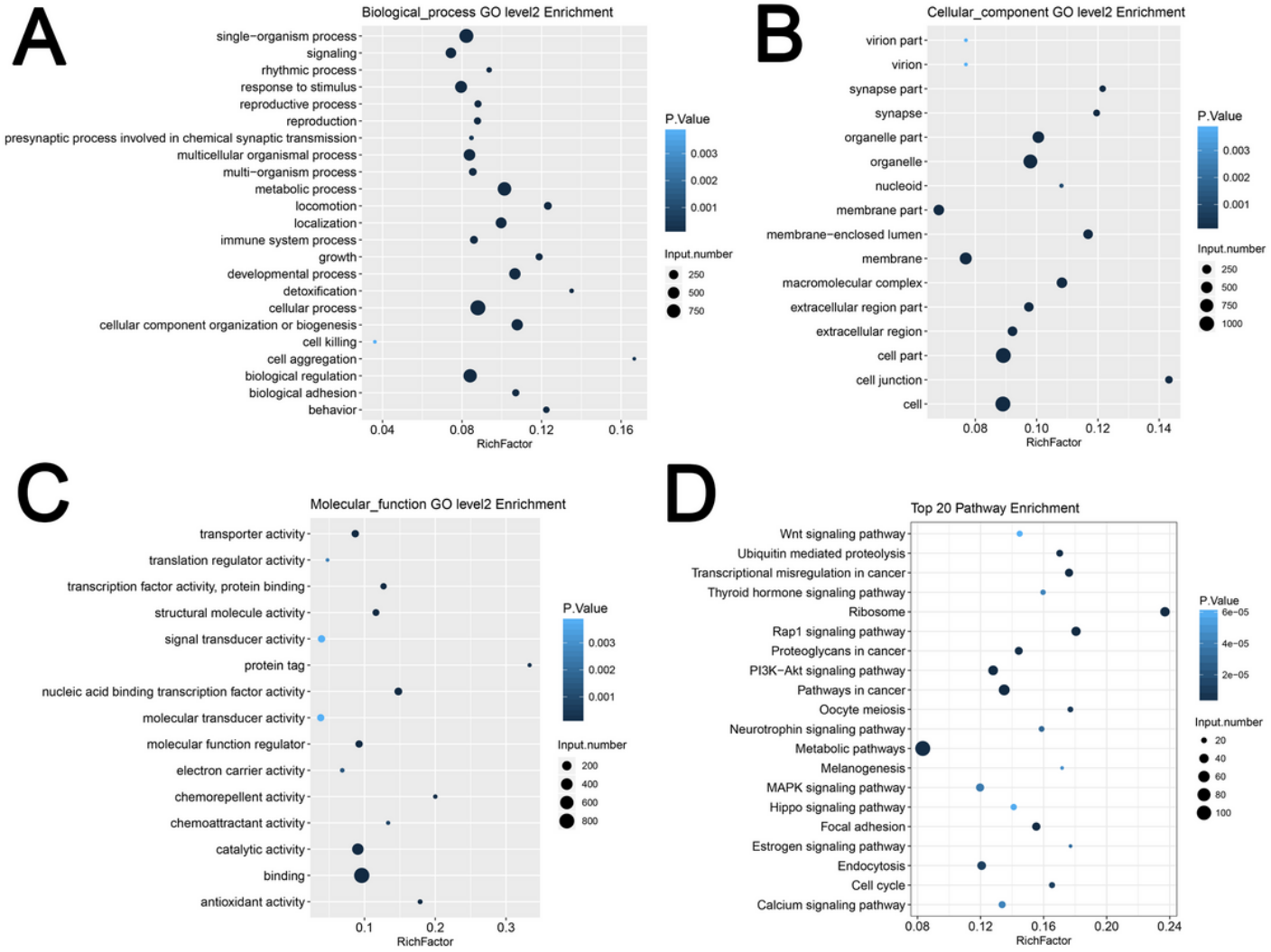

Figure 3

GO and KEGG analysis of differentially expressed mRNAs. (a) The 23 significantly different biological process terms revealed by GO analysis are shown. **(b)** The 16 molecular function terms with significant differences in the GO analysis are shown. **(c)** The 15 cellular component terms with significant differences in the GO analysis are shown. **(d)** The top 20 functional pathways of circRNA host genes were determined by KEGG enrichment analysis.

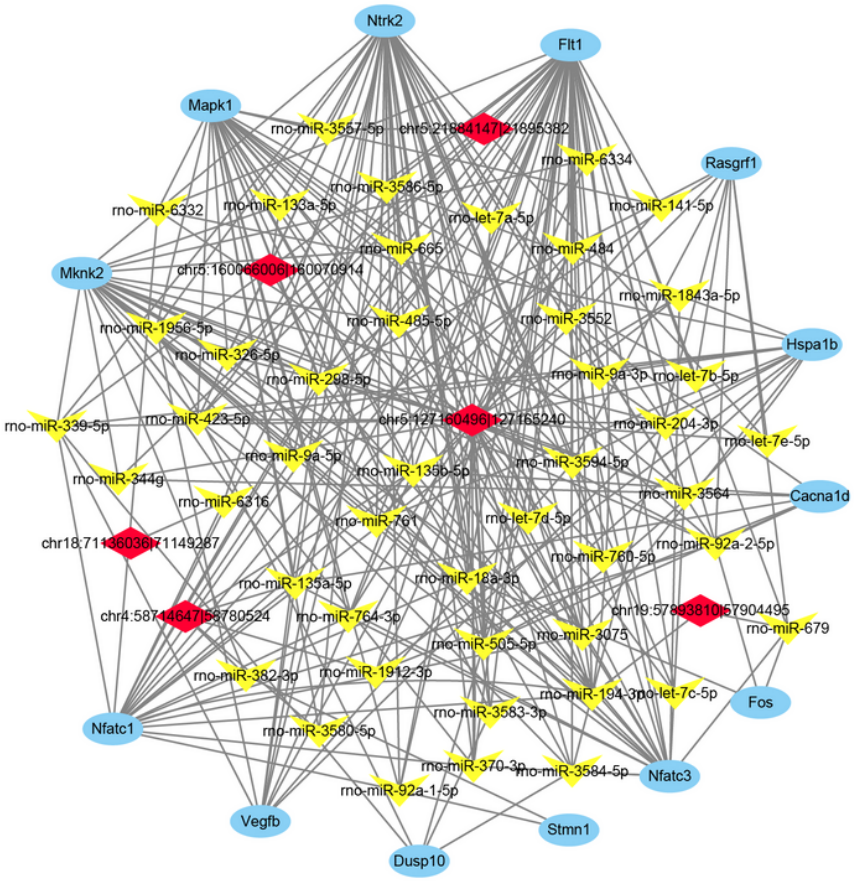

Figure 4

circRNA-miRNA-mRNA network of differentially expressed circRNAs and mRNAs in the MAPK signaling pathway. A circRNA-miRNA-mRNA network was constructed based on the MAPK signaling pathway. Six circRNAs, 46 miRNAs and 13 mRNAs were included. The blue ellipse indicates differentially expressed mRNA. The yellow arrowhead indicates predicted miRNA. The red quadrangle indicates differentially expressed circRNA. The black line indicates the relationship among these modules.

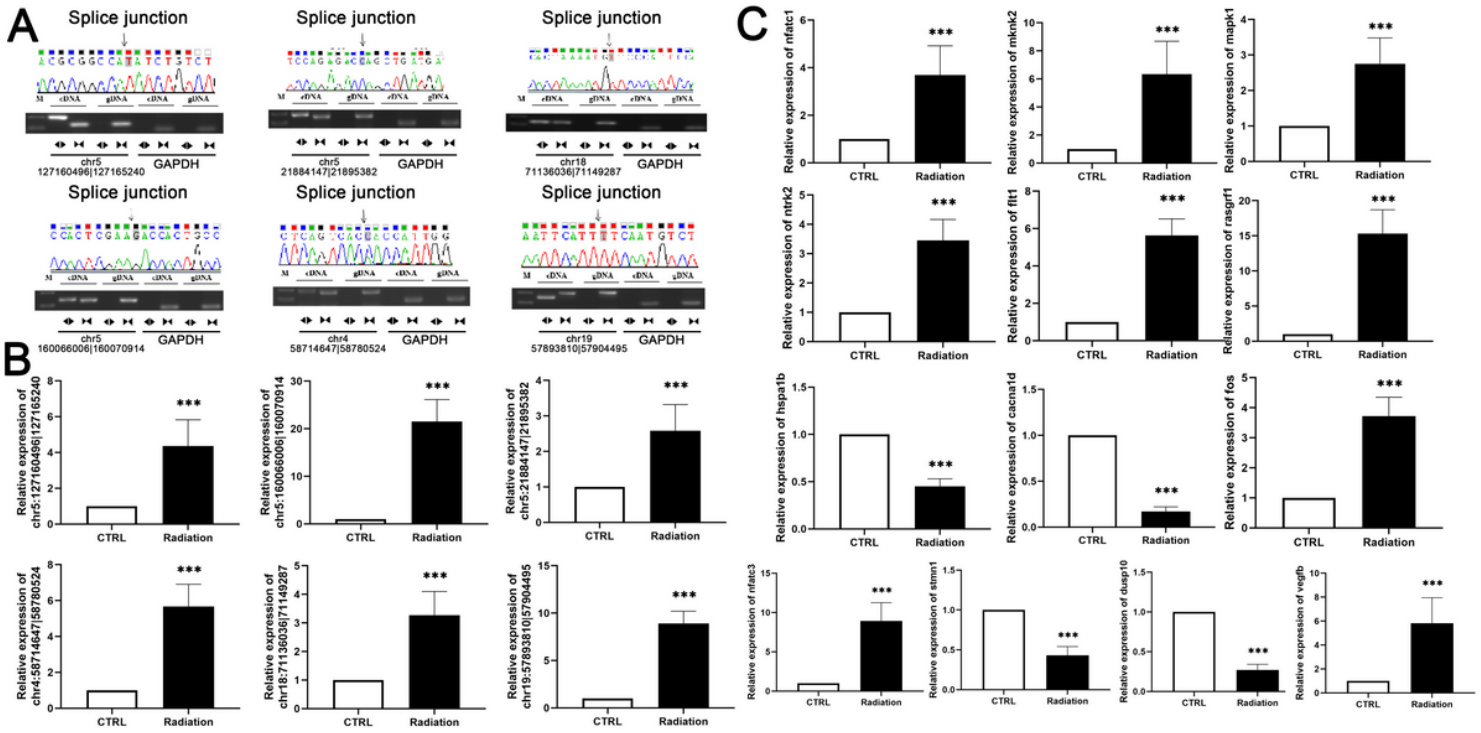

Figure 5

Validation of differentially expressed circRNAs and mRNAs in the MAPK signaling pathway. (a) RT-PCR and Sanger sequencing assays confirmed the circular form and conjunctive site of 6 circRNAs in the circRNA-miRNA-mRNA network. **(b)** qRT-PCR confirmed the differential expression of 6 circRNAs in the circRNA-miRNA-mRNA network. **(c)** qRT-PCR confirmed the differential expression of 13 mRNAs in the circRNA-miRNA-mRNA network. *** Indicates P < 0.001.

Supplementary Files

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